The Journal of Molecular Diagnostics, Vol. 13, No. 1, January 2011

DOI: 10.1016/j.jmoldx.2010.11.008

Multiplexed Methylation Profiles of Tumor Suppressor Genes in Bladder Cancer

Maria José Cabello,* Laura Grau,* Noreli Franco,* Esteban Orenes,* Miguel Alvarez,† Ana Blanca,‡ Oscar Heredero,§ Alberto Palacios,§ Manuel Urrutia,§ Jesus María Fernández,† Antonio López-Beltrán,‡ and Marta Sánchez-Carbayo*

From the Tumor Markers Group, Molecular Pathology Program, Spanish National Cancer Center, Madrid; the Urology Department,† Hospital Central de Asturias, Oviedo; the Pathology Department,‡ Hospital Reina Sofía, Córdoba; and the Urology Department,§ Hospital Universitario de Salamanca, Salamanca, Spain*

Changes in DNA methylation of tumor suppressors can occur early in carcinogenesis and are potentially important early indicators of cancer. The objective of this study was to assess the methylation of 25 tumor suppressor genes in bladder cancer using a methylation-specific (MS) multiplex ligation-dependent probe amplification assay (MLPA). Initial analyses in bladder cancer cell lines (n = 14) and fresh-frozen primary bladder tumor specimens (n = 31) supported the panel of genes selected being altered in bladder cancer. The process of MS-MLPA was optimized for its application in body fluids using two independent training and validation sets of urinary specimens (n = 146), including patients with bladder cancer (n = 96) and controls (n = 50). *BRCA1* (71.0%), *WT1* (38.7%), and *RARB* (38.7%) were the most frequently methylated genes in bladder tumors, with *WT1* methylation being significantly associated with tumor stage (P = 0.011). *WT1* and *PAX5A* were identified as methylated tumor suppressors. In addition, *BRCA1*, *WT1*, and *RARB* were the most frequently methylated genes in urinary specimens. Receiver operating characteristic curve analyses revealed significant diagnostic accuracies in both urinary sets for *BRCA1*, *RARB*, and *WT1*. The novelty of this report relates to applying MS-MLPA, a multiplexed methylation technique, for tumor suppressors in bladder cancer and body fluids. Methylation profiles of tumor suppressor genes were clinically relevant for histopathological stratification of bladder tumors and offered a noninvasive diagnostic strategy for the clinical management of patients affected with uroepithelial neoplasias. *(J Mol Diagn 2011, 13:29–40; DOI: 10.1016/j.jmoldx.2010.11.008)*

Bladder cancer can be described as a molecular disease, driven by the multistep accumulation of genetic and epigenetic factors.[1,2] Epigenetic alterations, including both DNA methylation and histone modifications, may result in silencing of cancer-related genes.[3–7] Alterations of DNA methylation patterns have been recognized as the most common epigenetic event in human cancers. Aberrant methylation of normally unmethylated dinucleotide guanine cytosine (CpG)-rich areas, also known as CpG islands, which are located in or near the promoter region of many genes, has been associated with transcriptional inactivation of important tumor suppressor, DNA repair, and metastasis inhibitor genes, among others.[8–12] Therefore, the detection of aberrant promoter methylation of cancer-related genes may be essential for diagnosis, prognosis, and/or detection of metastatic potential of tumors, including bladder cancer.[8–12] Because the number of genes known to be hypermethylated in cancer is large and increasing, sensitive and robust multiplexed methods for the detection of aberrant methylation of promoter regions are desirable.

Historically, the molecular pathogenesis of cancer has been teased out one gene at a time. The development of high-throughput profiling approaches accelerates the discovery of genetic and epigenetic events associated with tumorigenesis and tumor progression. The CpG arrays represent a high-throughput technology for the discovery of genes frequently hypermethylated during disease progression.[13,14] This technology has recently been applied to compare the methylation patterns of invasive bladder tumors with their respective normal urothelium counterparts.[15] The assessment of the methylation status of multiple genes is feasible using methylation-specific (MS)–derived techniques from multiplex ligation-dependent probe amplification (MLPA).[16] Methylation-specific multiplex ligation-dependent probe amplification is a PCR-based technique that allows the semiquantitative detection of changes in DNA promoter methylation of multiple genes (generally >30 sequences) in a single experiment.[16,17] Discrimination between methylated and

Supported by the Spanish Ministry of Science and Innovation grant SAF2009-13035 (M.S.-C.).

Accepted for publication July 20, 2010.

Supplemental material for this article can be found at *http://jmd.amjpathol.org* or at doi:10.1016/j.jmoldx.2010.11.008.

Address reprint requests to Marta Sánchez-Carbayo, Ph.D., Tumor Markers Group, 310A, Spanish National Cancer Research Center, Melchor Fernández Almagro 3, Madrid E-28029, Spain. E-mail: mscarbayo@cnio.es.

unmethylated targets is based on the annealing of probes containing a recognition site for the methylation-sensitive restriction enzyme HhaI. Methylation-specific multiplex ligation-dependent probe amplification has been applied to the multiplexed measurement of methylated genes in several diseases, including cancer.[18–27] Despite being explored in several neoplastic diseases, its potential utility in bladder cancer and body fluids has not been evaluated yet. In this study, we initially assessed, by MS-MLPA, whether a selected panel of candidate tumor suppressor genes could be methylated in bladder cancer cell lines and bladder tumors. Next, we determined whether it was possible to assess the methylation status of such genes in a multiplexed manner in urinary specimens to be used for bladder cancer diagnostics.

Materials and Methods

Bladder Cancer Cell Lines

Fourteen bladder cancer cell lines derived from transitional noninvasive (RT4, RT112, and 5637), invasive (UM-UC-3, J82, EJ138, and T24), metastatic (T24T, FL3, SFL4, and TCCSUP), and squamous (ScaBER) bladder tumors were obtained from the American Type Culture Collection, Rockville, MD; grown; and collected under standard tissue culture protocols, as previously described.[28] To test the linearity of the MS-MLPA assay, DNA isolated from the EJ138 bladder cancer line was methylated *in vitro* using SssI methylase, as described by the manufacturer (New England Biolabs, Ipswich, Australia). Methylated EJ138 cells were diluted to 50% methylated, using the original unchanged DNA. Two bladder cancer cell lines, RT4 and J82, were included in all sample runs to test the reproducibility of MS-MLPA.

Tumor Samples

The study cohort consisted of fresh-frozen untreated primary bladder specimens from 31 patients undergoing surgery as the primary treatment. Patients were serially collected during a 24-month period (July 2007 to July 2009). Primary bladder tumors were collected and handled anonymously, following ethical and legal protection guidelines of human subjects at participating hospitals. Tumor tissue was obtained intraoperatively and immediately frozen. The inclusion criteria of patients with newly diagnosed bladder cancer were based on histopathological information, covering from early to advanced stages. In addition, tissue material had to be available for obtaining high-quality DNA for methylation analyses. An initial series of 31 frozen bladder specimens served to screen MS-MLPA methylation rates among non–muscle-invasive tumors ($n = 19$) [ie, pTa ($n = 10$) and pT1 ($n = 9$)] and muscle-invasive cases ($n = 12$) [ie, pT2 ($n = 7$), pT3 ($n = 3$), and pT4 ($n = 2$)], defined under standard criteria.[29] The optimal cutting temperature compound-embedded tissues were macrodissected based on hematoxylin-eosin evaluations to ensure a minimum of 75% of tumor cells.[30] The demographic information of patients with bladder cancer indicated the presence of 29 men and 2 women (median age, 77 years; age range, 57–86 years).

Urinary Samples

The urine specimens of individuals presenting microscopic hematuria under first suspicion of bladder cancer were collected immediately before cystoscopy during a 24-month period (July 2007 to July 2009). Based on cystoscopic information, the individuals analyzed were classified into controls with different urological diseases (eg, urinary tract infections, lithiasis, and benign prostatic hyperplasia) and patients with bladder cancer. Healthy individuals without microscopic hematuria were also included. Samples were handled anonymously, following ethical and legal guidelines at participating hospitals. The presence of bladder cancer was confirmed by cystoscopy, the gold standard.[29] Urinary specimens ($n = 146$) served to analyze the clinical utility of MS-MLPA methylation at discriminating patients with bladder cancer ($n = 96$) from controls ($n = 50$), including healthy individuals and patients with benign urological diseases. Urine samples were prospectively collected and randomly divided into training and validation sets based on cystoscopy and histopathological information to obtain similar subsets of patients with bladder cancer and controls. In the training group, the demographic information of the control individuals indicated the presence of 18 men and 7 women (median age, 67 years; age range, 35–86 years), whereas in the patient group, there were 39 men and 8 women (median age, 75 years; age range, 36–90 years). Histopathological information after subsequent surgical interventions provided tumor stage distribution among patients with bladder cancer: pTa ($n = 13$), pT1 ($n = 25$), and pT2+ ($n = 9$). Tumor grade distribution was as follows: G1, $n = 15$; G2, $n = 14$; and G3, $n = 18$. Both of these variables were defined under standard criteria.[29] In the validation group, the demographic information of the control individuals indicated the presence of 15 men and 10 women (median age, 69 years; age range, 27–84 years); in the patient group, there were 42 men and 7 women (median age, 74 years; range, 47–99 years). Histopathological information after subsequent surgical interventions provided tumor stage distribution among patients with bladder cancer: pTa ($n = 15$), pT1 ($n = 25$), and pT2+ ($n = 9$). The tumor grade distribution was as follows: G1, $n = 15$; G2, $n = 16$; and G3, $n = 18$. Both of these variables were defined under standard criteria.[29]

DNA Extraction

Genomic DNA from tissue, cell lines, and urinary samples was extracted using standard methods. For tissue specimens, cryosection slides from opposite sides of frozen tumors were analyzed by hematoxylin-eosin staining. When the content of tumor cells was estimated to be greater than 75% on consecutive sections, corresponding pieces were digested using proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) overnight before DNA extraction. The concentration and purity of DNA

samples were determined with a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE). DNA quality was evaluated based on 260/280 ratios of absorbances, and the integrity was also checked by gel electrophoresis analysis using a bioanalyzer (Agilent 2100; Agilent Technologies, Palo Alto, CA).

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

The present study used an MS-MLPA probe set (ME002; MRC-Holland, Amsterdam, the Netherlands) that can simultaneously check for aberrant methylation at one or two CpG dinucleotides of the following proven or suspected tumor suppressor genes: *PTEN*, *MGMT* (2 probes: *MGMT* and *MGMT-2*), *CD44*, *WT1*, *GSTP1*, *ATM*, *IGSF4*, *STK11*, *CHFR*, *BRCA2*, *RB1* (2 probes: *RB1* and *RB1-2*), *THBS1*, *ASC*, *CDH13*, *TP53*, *BRCA1*, *TP73*, *GATA5*, *RARB*, *VHL*, *ESR1*, *PAX5A*, *CDKN2A*, and *PAX6*. Probe sequences, gene loci, and chromosome locations are available from MRC-Holland (Amsterdam, Netherlands). Some of the genes were represented by two probes that each recognized a different HhaI restriction site in the promoter region of the respective genes. The experimental procedure was performed, and results were analyzed according to the manufacturer's instructions, with minor modifications. In short, DNA (200 ng) was dissolved in up to 5 μL of Tris(hydroxymethyl)-aminomethane ethylenediamine tetra-acetic acid buffer (10-mmol/L Tris, pH 8.2; and 1-mmol/L EDTA, pH 8.0), denatured, and subsequently cooled to 25°C. After adding the probe mix, the probes were allowed to hybridize (for 16 hours at 60°C). Subsequently, samples were divided into two: half of the sample was ligated, and for the other part of the sample, ligation was combined with the HhaI digestion enzyme. This digestion resulted in ligation of only the methylated sequences. PCR was performed on both parts of the samples in a volume of 50 μL containing 10 μL of the ligation reaction mixture using a thermal cycler (MJ Research Inc, Waltham, MA), with 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 20 minutes. Aliquots of 2 μL of the PCR reaction were combined with 0.12 μL of LIZ500-labeled internal size standard (Applied Biosystems, Foster City, CA) and 9.0 μL of deionized formamide. After denaturation, fragments were separated and quantified by electrophoresis on a capillary sequencer (ABI 3700; Applied Biosystems); and data were analyzed with software (Peak Scanner v1.0; Applied Biosystems). Peak identification, values corresponding to peak size (in bp), and peak areas were used for further data processing. Automated fragment and data analysis was performed by exporting the peak areas to an Excel-based analysis program (Coffalyser V8; MRC-Holland). For hypermethylation analysis, the "relative peak value" or the so-called probe fraction of the ligation-digestion sample is divided by the relative peak value of the corresponding ligation (undigested) sample, resulting in a so-called methylation ratio. Aberrant methylation was scored when the calculated methylation ratio was 0.30 or greater, corresponding to 30% of methylated DNA. The methylated ratios

Table 1. Methylation Profiles of 14 Bladder Cancer Cell Lines*

Gene name	RT4	RT112	5637	J82	UM-UC-3	EJ138	HT1376	HT1997	T24	T24T	FL3	SLF4	TCCSUP	ScaBER
TP73†	0.19	0.59	0.08	0.12	**0.76**	0.03	0.07	0.16	0.11	0.01	0.05	0.02	0.06	**0.94**
MSH6†	0.21	0.15	0.27	0.26	1	0.07	0.11	0.18	0.12	0.23	*0.39*	0.28	*0.42*	0.63
VHL	0.18	0.06	0.19	0.21	0.52	0.02	0.04	0.18	0.15	0.04	0.11	0.03	0.04	0.07
RARB	0.07	1	0.61	0.17	*0.37*	*0.3*	0.11	0.7	*0.39*	*0.42*	0.07	*0.33*	**0.73**	**0.95**
ESR1†	1	1	0.13	0.03	1	1	0.12	**0.95**	**0.8**	1	1	1	1	*0.3*
CDKN2A	0.7	1	0.11	0.04	*0.37*	0.02	0	0.08	0.01	0.02	0.22	0	0.01	0.09
PAX5A†	0.19	0.56	0.23	0.22	1	0.62	**0.73**	0.53	**0.73**	1	1	1	1	0.55
PTEN†	0.05	0	0.06	0.05	*0.37*	0.02	0.08	0.08	0.02	0.04	0.03	0.02	0.03	0.09
MGMT	0.07	0.05	*0.39*	0.2	1	0.05	0.07	0.04	0.02	0.04	0.04	0.06	0.03	0.58
MGMT-2	0.22	0.03	*0.39*	0.29	1	0.66	0.1	0.1	*0.49*	0.06	0.22	0.16	0.11	1
PAX6	0.55	0.67	0.08	0	0	0	0	0.61	0	0.02	0	0.02	0.01	1
WT1†	1	**0.77**	0.11	0.59	0	0.63	0.06	1	**0.84**	**0.85**	1	**0.84**	1	1
CD44†	*0.43*	0.61	0.09	1	0	0.02	**0.72**	0	0.04	0.03	0.2	0.1	0.12	0.25
GSTP1	1	0.09	0.05	0.05	0.15	0	0.08	0.09	0.02	0.02	*0.31*	0.06	0.02	0.22
ATM†	0.03	0.04	0.1	0.08	0	0	0.07	0.04	0.02	0	0.02	0.04	0.02	0.23
IGSF4†	0.02	*0.49*	0.19	*0.34*	0.11	*0.4*	0.12	0.14	*0.41*	0.03	0.06	0.07	0.09	0.51
CHFR†	0.04	0	0	0.1	0	0.02	0.05	1	0.05	0.02	0.08	0.01	0.02	*0.43*
BRCA2†	0.1	0.03	0.14	0	0	0.02	0.03	0.05	0.09	0.03	*0.42*	0.02	0.07	0.12
RB1	0.02	0.02	0.06	0.02	0.04	0.01	0.05	0.02	0.03	0.03	0.06	0.06	0.04	*0.35*
RB1-2	0.05	0.14	0.13	0.2	0	0.16	0.07	0.03	0.06	0.03	0.11	0.08	0.08	0.21
THBS1†	0.45	0.24	0	*0.32*	0	0.13	0	0.18	*0.39*	0.2	*0.43*	0.11	0.12	0.19
PYCARD†	0.64	0.1	0.16	0.1	1	**0.84**	0.05	0.02	0.01	*0.48*	1	**0.83**	0.63	*0.34*
CDH13	0.6	0.25	0.1	1	1	1	0.12	**0.98**	0.02	0.67	1	*0.36*	0.62	0.17
TP53	0.04	0.52	0.21	0.14	*0.31*	0.11	0.5	0.22	0.07	0.1	0.08	0.04	0.12	0.19
BRCA1	**0.71**	1	1	1	0.57	0.13	1	0.21	0.17	0.2	0.18	0.05	0.11	0.28
STK11†	0.08	0	*0.31*	0.06	0.17	0.01	0.06	0.22	0.04	0.02	0.2	0.05	0.02	0.22
GATA5†	1	0.04	0.59	**0.81**	0.57	1	0.05	1	1	**0.86**	1	1	1	1

*Methylation ratios were interpreted as follows: absence of hypermethylation, 0.00 to 0.29; mild hypermethylation, 0.30 to 0.49 (italicized); moderate hypermethylation, 0.50 to 0.69 (underlined); and extensive hypermethylation, greater than 0.70 (boldfaced and underlined).
†Novel candidates not reported to be methylated in bladder cancer to date.

were interpreted as absence of hypermethylation (0.00 to 0.29), mild hypermethylation (0.30 to 0.49), moderate hypermethylation (0.50 to 0.69), and extensive hypermethylation (>0.70). In genes with more than one probe, ratios were calculated independently for methylation analysis.

Statistical Analysis

Associations among MS-MLPA and tumor stage and grade were evaluated using nonparametric Wilcoxon-Mann-Whitney and Kruskall-Wallis tests. Adjustment of *P* values for multiple comparisons, estimating their 99% confidence interval (CI), was assessed using Monte Carlo bootstrapping with 10,000 iterations using computer software (SPSS 18.0.1 for Windows 2010; SPSS Inc, Chicago, IL).[31] Associations between methylation candidates were analyzed using Kendall's τ test, including 300 pairs of pairwise bootstrapping iterations to adjust for multiple comparisons.[31] To estimate the sensitivity and specificity of each probe of the assay, methylation was scored when the calculated methylation ratio was 0.30 or greater. The bladder cancer diagnostic utility of the MS-MLPA assay was initially evaluated in a training set of 47 patients with bladder cancer and 25 individuals without evidence of disease, as confirmed by cystoscopy, the gold standard. The bladder cancer diagnostic utility of the MS-MLPA assay was then evaluated in a testing set of 49 patients with bladder cancer and 25 individuals without evidence of disease, as confirmed by cystoscopy. The receiver operating characteristic curve analyses defined the diagnostic performance of methylation in urinary specimens, given by the area under the curve, estimating its 95% CI.[29] Bilateral significance was assessed by adjusting *P* values for multiple comparisons estimating their 99% CI, using Monte Carlo bootstrapping with 10,000 iterations. All statistical analyses were performed using software (SPSS 18.0.1 for Windows 2010).

Results

Quality Assessment of the MS-MLPA Assay

To test the linearity of the MS-MLPA assay, a titration study was performed using the *in vitro* methylated bladder cancer cell line EJ138. Changes in methylation ratios between unmodified and *in vitro* methylated DNA were evaluated for each candidate. Methylated genes remained hypermethylated after 100% *in vitro* modifications, with minor changes (media, 14.8%; SD, 13.5%; range, 0% to 37%). More important, unmethylated genes showed at least a 33.0% increase in methylation (media, 78.9%; SD, 22.9%; range, 33% to 100%), as shown in Supplemental Table S1A at *http://jmd.amjpathol.org*. This titration experiment revealed high recoveries for the probes analyzed and suggested that the assay is semiquantitative. Thus, methylation ratios could be considered indicative of the amount of methylated DNA. To test the reproducibility of the assay, two bladder cancer cell lines (ie, RT4 and J82) were included as control samples in each assay run. The coefficients of variation of the methylation ratios of these replicated experiments of the different cells analyzed are shown in Supplemental Table S1B at *http://jmd.amjpathol.org*. The thresholds previously defined for methylation detection suggested high repro-

Table 2. Methylation Profiles of Bladder Tumors*

Gene name	PTaG1 98THO	PTaG1 90TM	PTaG1 95TM	PTaG1 86TM	PTaG1 93TM	PTaG1 91TM	PTaG1 89TM	PTaG1 92TM	PTaG1 100TM	PTaG2 118TH	PT1G1 85TM	PT1G1 94TM	PT1G1 99TM	PT1G2 101TM	PT1G2 105THO
TP73†	0.03	*0.31*	0.11	0.19	0.05	0.01	0.02	0.05	0.01	0.02	0.07	0.03	0.04	0.15	0
MSH6†	0.14	0.29	*0.37*	*0.32*	0.19	0.13	0.14	0.1	0.5	0.27	0.12	0.14	*0.31*	0.26	0.03
VHL	0	1	0.64	0.19	0.08	0.08	0.04	*0.36*	*0.45*	0	0	0.23	0	0.28	0
RARB	0.36	0	0.5	0.27	0.27	0	0.06	0.17	1	0.17	0.1	0.39	**0.99**	0	0
ESR1†	0.2	*0.4*	0.07	0.11	0.15	0.03	0.04	0.28	0.07	0.27	0.1	0.04	0.14	0.04	0
CDKN2A	0.05	0	0	0.03	0.01	0.01	0.02	0.02	0	0.14	0	0	0	0.1	0
PAX5A†	0.09	0	0.05	0.09	0.03	0.14	0.08	0.02	0.07	0.04	0.08	0.07	0.21	0.05	0.66
PTEN†	0	0.21	0.09	0.07	0.01	0.04	0.05	0	0.02	0.09	0.07	0.05	0.03	0.09	0
MGMT	0	0	0.05	0.06	0.01	0.03	0.02	0.04	0.08	0.02	0.08	0.04	0	0.15	0.24
MGMT-2	0.07	0.64	0.08	*0.38*	0.08	0.05	0.05	0.05	0.12	0.1	0.13	0.11	0.05	0.25	0
PAX6	0	0	0	0.13	0.02	0	0.01	0.03	0	0	0	0	0.21	0.06	0
WT1†	0.15	0.65	0.11	0.14	0.1	0.04	**0.87**	0.16	**0.98**	0.05	*0.39*	0.19	0.68	0.12	1
CD44†	0.13	0	0	0.07	0.07	0	0	0.06	0	0	0.05	0	0	**0.88**	0
GSTP1	0.05	0	0.18	0.06	0.04	0.02	0.02	0	0	0.01	0.06	0.04	0	0	0
ATM†	0.01	0.21	0.03	0.04	0.02	0.01	0.01	0.01	0.02	0	0.02	0	0	0.01	0.08
IGSF4†	0.27	0.54	*0.31*	0.2	0.13	0.06	0.08	0.09	*0.34*	0.15	0.09	0.18	0.68	*0.33*	*0.3*
CHFR†	0.04	0	0	0	0	0.08	0	0	0.12	0.11	0	0	0.24	0	0
BRCA2†	0	0	0	0.02	0.04	0.01	0.04	0.06	0.09	0	0	0.01	0.1	0.13	0
RB1	0.03	0	0.03	0.03	0.01	0.02	0.01	0.02	0.07	0.04	0.03	0.02	0.07	0.03	0.02
RB1-2	0	0	0.18	0.12	0.1	0.06	0.08	0.09	0.1	0.14	0	0.07	0.08	0.21	*0.43*
THBS1†	0.22	1	0.21	0.15	*0.48*	0.23	0.17	0.07	0.22	0.17	*0.47*	0.5	0.16	*0.36*	0
PYCARD†	0	0	0	0.01	0	0.01	0	0	0	0	0	0.02	0	0	0
CDH13	0.17	0	0	0.18	0.13	0.04	0.09	0.19	**0.97**	0	0.55	0.29	0.19	0.16	0
TP53	0.09	0.74	0.75	0.13	0.12	0.07	0.17	0.1	0.16	0.13	0.26	0.14	0.16	0.13	*0.4*
BRCA1	0.17	1	1	**0.85**	1	1	*0.45*	1	*0.48*	0.67	1	1	1	1	0
STK11†	0	0	*0.2*	0.06	0.01	0.01	0	0.04	0.25	0.02	0.08	0	0.06	0.04	0
GATA5†	0.25	0	*0.33*	0.16	0.05	0.02	0.03	0.06	**0.92**	0.04	0.19	0.09	**1**	0.08	0.26

(*table continues*)

*Methylation ratios were interpreted as follows: absence of hypermethylation, 0.00 to 0.29; mild hypermethylation, 0.30 to 0.49 (italicized); moderate hypermethylation, 0.50 to 0.69 (underlined); and extensive hypermethylation, greater than 0.70 (boldfaced and underlined).

†Novel candidates not reported to be methylated in bladder cancer to date.

ducibility of the methylation profiles. Overall, analysis of these bladder cancer cell lines revealed reproducible results, allowing methylation assessment for clinical routine practice using the selected panel of candidate genes.

MS-MLPA Profiles of Bladder Cancer Cell Lines

The methylation profiles of the 25 genes being studied were initially tested in 14 bladder cancer cell lines derived from bladder tumors covering early lesions and late tumors along the progression of the disease. In Table 1, an overview of the methylation patterns of these cell lines is shown, ordered based on the increasing stage of the tumors from which these bladder cancer cell lines were derived from. Seven genes (ie, *GATA5*, *STK11*, *RARβ*, *PAX6*, *CDKN2A*, *PYCARD*, and *THSB1*) were found methylated in more than 50% of the cell lines covering various stages of progression. With the exception of four genes methylated in cells that were unmethylated in the bladder tumors, most of the genes methylated in the cell lines were also methylated in the bladder tumor specimens analyzed, as shown later. More important, the number of methylated genes and the degree of hypermethylation increased according to the aggressiveness of the cells. With the exception of *GSTP1*, all of the genes being studied were methylated in at least one of the cell lines analyzed. These initial analyses suggested that the panel of candidate genes selected could be appropriate for detecting aberrant methylation profiles in human bladder cancer specimens.

MS-MLPA Profiles of Bladder Tumors

In the next step, we tested whether MS-MLPA could be applied to bladder tissues. The genes more frequently methylated in noninvasive bladder lesions were *WT1* and *BRCA1*, whereas the most commonly methylated genes in muscle-invasive bladder tumors were *BRCA1*, *RARB*, and *THSB1* (Table 2). Overall, the most frequent promoter of hypermethylation by MS-MLPA was identified for the *BRCA1* gene, followed by the *RARB* and *WT1* genes (Table 3). Promoter hypermethylation of genes previously reported as methylated in bladder cancer included *RARB*, *CDH13*, and *BRCA1* (Table 3). When comparing methylation rates in bladder tumors depending on histopathological variables, it was observed that the methylation of *WT1* was significantly associated with tumor stage ($P = 0.025$; 99% CI, 0.021 to 0.029) and that the methylation of *VHL* ($P = 0.022$; 99% CI, 0.018 to 0.025), *MGMT* ($P = 0.030$; 99% CI, 0.025 to 0.034), and *BRCA1* ($P = 0.017$; 99% CI, 0.014 to 0.020) was significantly associated with tumor grade. This set of analyses suggested that the panel of candidate genes selected could be clinically relevant for histopathological staging of human bladder tumors.

MS-MLPA Profiles in Matching Urinary Specimens and Bladder Tumors

Once the MS-MLPA assay was shown applicable to bladder tissue specimens, we tested the possibility of applying the assay to noninvasive urinary genomic DNA. Initially, the technique was tested in urine specimens of patients for whom MS-MLPA had been performed in

Table 2. *Continued*

PT1G3 99THO	PT1G3 106THO	PT1G3 7021T	PT1G3 6885T	PT2G3 1018T	PT2G3 87TM	PT2G3 96TM	PT2G3 97TM	PT2G3 84THO	PT2G3 92THO	PT2G3 113TH	PT3bG3 7303T	PT3bG3 6599T	PT3bG3 6093T	PT4G3 98TM	PT4G3 124THO
0.06	0.03	0.02	0.14	0.08	0.13	0.05	0	0.01	0.16	0	0.06	0.08	0.03	0.52	0.11
0.41	0.02	0.03	*0.34*	*0.35*	0.23	*0.42*	0.2	0.15	0.26	*0.36*	0.2	0.13	0.19	0.23	0.18
0.05	0.04	0	0	0.02	0.14	0.1	0	0.05	0.04	0.02	0	0.03	0.04	0	0
0.11	0.55	1	1	0.13	0.05	0.12	1	*0.37*	0	0	0	1	1	0.13	1
0.04	*0.31*	0.02	0.16	0.13	0.09	0.57	0	0	0.13	0.03	0.06	0.22	0.23	0.62	1
0.05	0	0	0.07	0.01	0.01	0.06	0	0.1	0	0.03	0	0.04	0	0	0
0.12	0.19	0.08	*0.31*	0.06	0.03	0.05	0.58	0.15	0.09	0	*0.31*	0.09	0.26	0.19	0
0.02	0.05	0.02	0.11	0.03	0.03	0.08	0.08	0.1	0	0	0.04	0.02	0.01	0.04	0
0.06	0.09	0.2	0.12	0.08	0.03	0.11	0	0.19	0.06	0.01	0.05	0.03	0.1	0.04	0.03
0.09	0.12	*0.38*	0.25	0.2	0.09	0.34	0.08	0.17	0.07	0.14	0.05	0.16	0.16	*0.34*	0.16
0	0.23	0.04	0	0.03	0	0.15	0	0	0	0	0	0	0	0	0
0.11	0.84	0.73	0.75	0.07	0.03	0.09	0	0.05	0.09	0.62	*0.45*	0.07	0.55	0.23	0.02
0.97	0	0.04	0	0	0.21	0.2	0	0.17	*0.31*	0	0	0.1	0.11	0.08	0
0.02	0.04	0	0.03	0.03	0.04	0.21	0	0	0.04	0	0.02	0.06	0.02	0.03	0
0.04	0.07	0.04	0.06	0.01	0.02	0.04	0	0.07	0.01	0.04	0.08	0.05	0.01	0.01	0.06
0.14	0.14	0.21	0.7	0.05	0.14	0.24	0.59	0.14	0.18	*0.31*	0.17	0.07	0.14	0.04	0.28
0	0.04	0.03	0.05	0.01	0.11	0.18	0	0.11	0.15	0	0.23	0.01	0	0.08	0
0.05	0.1	0.01	0.05	0.05	0.11	0	0.52	0.16	0.04	0.04	0.02	0.01	0.04	0.07	0
0.06	0.06	0.03	0	0.02	0.02	0.09	0.06	0.03	0.03	0.01	0.02	0.02	0.02	0.03	0.01
0.11	0	0	0.04	0.02	0.05	0.16	0	0.1	0.14	0.04	0.1	0.02	0.04	0.07	0
0.08	0.15	1	0.15	0.16	*0.35*	0	1	0.5	*0.4*	*0.39*	0.07	0.12	0.26	*0.22*	0
0.02	0.04	0	0.03	0.03	0.01	0.23	0	0	0	0	0	0	0.06	0	0
0.11	1	0.31	0.06	0.13	0.08	0.2	0.84	0	0.21	*0.35*	0.19	0.2	0.57	*0.39*	0.15
0.04	0.23	0.04	0.07	0.05	0.22	0.2	0.61	0.09	0.05	0.16	0.04	0.03	0.08	0.1	0.1
0.06	*0.3*	0.28	*0.39*	0.15	1	1	1	1	*0.33*	0.23	*0.37*	0.24	0.24	1	0.07
0.05	0.05	0.03	0	0.01	0.18	0.21	0	0	0	0.11	0.07	0	0.03	0.06	0
0.06	0.52	0.08	1	0.04	0.09	0.25	0.25	0.06	0.13	1	0	0.25	*0.42*	*0.34*	0

Table 3. Summary of the Frequency of Methylation of the Genes Under Study in Bladder Cancer Cells and Tumor Specimens

	Bladder cancer cells (n = 14)		pTas (n = 10)		pT1 (n = 9)		pT2+ (n = 12)		All tumors (n = 31)	
Gene name	No. of samples*	F value†	No. of samples*	F value†	No. of samples*	F value†	No. of samples*	F value†	No. of samples*	F value†
TP73‡	3	21.43	1	10.00	0	0.00	1	8.33	2	6.45
MSH6‡	4	28.57	3	30.00	3	33.33	3	25.00	9	29.03
VHL	1	7.14	4	40.00	0	0.00	0	0.00	4	12.90
RARB	11	78.57	3	30.00	5	55.56	5	41.67	12	38.71
ESR1‡	3	21.43	1	10.00	1	11.11	3	25.00	5	16.13
CDKN2A	11	78.57	0	0.00	0	0.00	0	0.00	0	0.00
PAX5A‡	1	7.14	0	0.00	2	22.22	2	16.67	4	12.90
PTEN‡	3	21.43	0	0.00	0	0.00	0	0.00	0	0.00
MGMT	5	35.71	0	0.00	0	0.00	0	0.00	0	0.00
MGMT-2	4	28.57	2	20.00	1	11.11	2	16.67	5	16.13
PAX6	11	78.57	0	0.00	0	0.00	0	0.00	0	0.00
WT1‡	4	28.57	3	30.00	6	66.67	3	25.00	12	38.71
CD44‡	2	14.29	0	0.00	2	22.22	1	8.33	3	9.68
GSTP1‡	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
ATM‡	5	35.71	0	0.00	0	0.00	0	0.00	0	0.00
IGSF4‡	2	14.29	3	30.00	4	44.44	2	16.67	9	29.03
CHFR‡	1	7.14	0	0.00	0	0.00	0	0.00	0	0.00
BRCA2‡	1	7.14	0	0.00	0	0.00	1	8.33	1	3.23
RB1‡	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
RB1-2‡	4	28.57	0	0.00	1	11.11	0	0.00	1	3.23
THBS1‡	8	57.14	2	20.00	4	44.44	5	41.67	11	35.48
PYCARD‡	9	64.29	0	0.00	0	0.00	0	0.00	0	0.00
CDH13‡	3	21.43	1	10.00	3	33.33	4	33.33	8	25.81
TP53‡	6	42.86	2	20.00	1	11.11	1	8.33	4	12.90
BRCA1‡	1	7.14	9	90.00	6	66.67	7	58.33	22	70.97
STK11‡	12	85.71	0	0.00	0	0.00	0	0.00	0	0.00
GATA5‡	14	100.00	2	20.00	3	33.33	3	25.00	8	25.81

*Those displaying a methylation ratio higher than 0.3.
†Percentual frequency within each group of specimens under analyses.
‡Novel candidates not reported to be methylated in bladder cancer to date.

matching tissue specimens. The percentual frequency of urine methylation found in matching tumor DNA was evaluated for the five pairs of available tumor DNA and matching urinary DNA (see Supplemental Table S2 at *http://jmd.amjpathol.org*). Methylation in urinary specimens was accompanied by methylation in matching tumor DNA for 50.0% of the genes found methylated in the urinary specimens. Among genes methylated in bladder tumors, 57.9% were also methylated in matching urinary specimens. With these few exceptions, in which methylation rates were close to the selected cutoff, high concordance was found for unmethylated genes between urine samples and matching tumor specimens. These analyses revealed that the methylation observed in bladder tumors was detectable in matching body fluids. Overall, these observations supported the idea that methylation profiles of urinary specimens could mimic bladder tumors and be used for diagnostic purposes. This set of analyses suggested that the panel of genes selected could estimate promoter methylation in urinary specimens for bladder cancer diagnostics.

Diagnostic Utility of MS-MLPA Profiles in the Training Set of Urinary Specimens

Once the feasibility of the genomic urinary DNA was evaluated, the MS-MLPA assay was then performed in 146 urinary specimens. A full series of control and bladder cancer urinary samples was then tested and divided into training and validation sets. The training set included 25 control individuals without bladder cancer (confirmed by cystoscopy) and 47 bladder tumors composed of non–muscle-invasive lesions (n = 38) and muscle-invasive bladder tumors (n = 9). An overview of methylation profiles of the training set showing the methylation ratios for all genes in each individual urinary specimen is displayed in Supplemental Table S3 at *http://jmd.amjpathol.org*. In the training set, the summary of the specificity in control specimens and the sensitivity in patients with bladder cancer is provided in Table 4. Methylation of several genes was simultaneously present, as revealed by Kendall's τ correlations, shown in Supplemental Table S4A at *http://jmd.amjpathol.org*. Non–muscle-invasive carcinomas revealed frequent promoter methylation (ie, in >35% of cases) of *BRCA1* [22 (57.9%) of 38], *RARB* [19 (50.0%) of 38], and *THBS1* [14 (36.8%) of 38]. In invasive bladder tumors, frequent promoter methylation was detected for *BRCA1* [6 (66.7%) of 9] and *CDH13*, *THBS1*, and *WT1* [all with 4 (44.4%) of 9]; *BRCA1* was the most frequently methylated in both tumor histosubtypes, with methylation in 59.6% of all bladder tumors. Methylation of *WT1* and *CDH13* was higher in muscle-invasive bladder tumors, whereas *RARB* and *PAX5A* methylation was more common in non–muscle-invasive bladder neoplasms compared with non–muscle-invasive lesions (Table 4). Although they were not among the most frequently

Table 4. Summary of the Sensitivity and Specificity of the Methylation Profiles in the Training Set of Urinary Specimens

	Controls (n = 25)		Patients (n = 47)		pTa (n = 13)		pT1 (n = 25)		pT2+ (n = 9)		G1 (n = 15)		G2 (n = 14)		G3 (n = 18)	
Gene name	No. of samples*	Specificity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity
TP73†	0	100	5	10.6	0	0	5	20	0	0	0	0	2	14.3	3	16.7
MSH6†	6	76	15	31.9	5	38.5	8	32	2	22.2	6	40	6	42.9	3	16.7
VHL	0	100	4	8.5	1	7.7	1	4	2	22.2	2	13.3	0	0	2	11.1
RARB	2	92	22	46.8	4	30.8	17	68	3	33.3	4	26.7	8	57.1	10	55.5
ESR1†	0	100	8	17.2	3	23.1	5	20	0	0	1	6.7	4	28.6	3	16.7
CDKN2A	0	100	4	8.5	0	0	4	16	0	0	1	6.7	0	0	3	16.7
PAX5A†	1	96	14	29.8	3	23.1	9	36	2	22.2	5	33.3	5	35.7	4	22.2
PTEN†	1	96	2	4.3	1	7.7	1	4	0	0	0	0	1	7.1	1	5.5
MGMT	0	100	3	6.4	1	7.7	1	4	1	11.1	1	6.7	1	7.1	1	5.5
MGMT-2	4	84	12	25.5	5	38.5	4	16	3	33.3	4	26.7	5	35.7	3	16.7
PAX6	0	100	2	4.3	1	7.7	1	4	0	0	1	6.7	0	0	1	5.5
WT1†	0	100	16	34	5	38.5	7	28	4	44.4	4	26.7	5	35.7	7	28.9
CD44†	3	88	12	25.5	2	14.3	10	40	0	0	4	26.7	2	14.3	6	33.3
GSTP1	0	100	4	8.5	1	7.7	3	12	0	0	1	6.7	2	14.3	1	5.5
ATM†	0	100	6	12.8	2	15.4	3	12	1	11.1	2	13.3	1	7.1	3	16.7
IGSF4†	0	100	8	17.2	2	15.4	5	20	1	11.1	3	20	1	7.1	4	22.2
CHFR†	0	100	3	6.4	0	0	3	12	0	0	1	6.7	0	0	2	11.1
BRCA2†	0	100	6	12.8	3	23.1	3	12	0	0	1	6.7	3	21.4	1	5.5
RB1	0	100	2	4.3	0	0	2	8	0	0	0	0	1	7.1	1	5.5
RB1-2	1	96	4	8.5	1	7.7	3	12	0	0	1	6.7	0	0	2	11.1
THBS1†	1	96	18	38.3	6	42.9	8	32	4	44.4	6	40	5	38.5	7	28.9
PYCARD†	0	100	2	4.3	1	7.7	1	4	0	0	1	6.7	0	0	1	5.5
CDH13	2	92	15	31.9	6	42.9	5	20	4	44.4	4	26.7	6	42.8	5	26.3
TP53	4	84	11	23.4	3	23.1	8	32	0	0	5	33.3	2	14.3	4	22.2
BRCA1	3	88	28	59.6	8	57.1	14	56	6	66.7	8	53.3	9	64.3	11	61.1
STK11†	0	100	2	4.3	0	0	2	8	0	0	0	0	0	0	2	11.1
GATA5†	1	96	7	14.9	1	7.7	4	16	2	22.2	2	13.3	2	14.3	3	16.7

*Those displaying a methylation ratio higher than 0.3.
†Novel candidates not reported to be methylated in bladder cancer to date.

methylated genes, a significant association was found between tumor stage and the methylation rates of *ATM* (P = 0.010; 99% CI, 0.007 to 0.012) and *MGMT-2* (P = 0.012; 99% CI, 0.009 to 0.015). Regarding tumor grade, the more frequent methylated genes in G1 tumors were *BRCA1*, *THBS1*, and *MSH6*, whereas in both G2 and G3 tumors, the most common methylated genes were *BRCA1* and *RARB*. Although it was not among the most frequently methylated genes, a significant association was found between *PAX6* urinary methylation rates and tumor grade (P = 0.023; 99% CI, 0.019 to 0.027). The receiver operating characteristic curve analyses supported the highest diagnostic accuracies for *BRCA1* (P = 0.002; 99% CI, 0.001 to 0.004), *RARB* (P = 0.007; 99% CI, 0.005 to 0.010), *CDH13* (P = 0.011; 99% CI, 0.008 to 0.014), and *WT1* (P = 0.017; 99% CI, 0.013 to 0.020) (Table 5 and Figure 1A).

Diagnostic Utility of MS-MLPA Profiles in the Validation Set of Urinary Specimens

An independent set of urinary specimens composed of 25 control individuals without bladder cancer and 49 patients with noninvasive bladder lesions (n = 40) and invasive bladder tumors (n = 9) was used to validate the clinical diagnostic value of the methylation profiles in an independent set of urinary samples (see Supplemental Table S5 at *http://jmd.amjpathol.org*). The summary of the specificity in control specimens and the sensitivity in the patients with bladder cancer of the validation set is provided in Table 6. Methylation of several genes was simultaneously present in urinary samples, part of them also observed in the training set, as revealed by Kendall's τ correlations in Supplemental Table S4B at *http://jmd.amjpathol.org*. Non–muscle-invasive carcinomas revealed frequent promoter methylation (ie, in >35% of cases) for *BRCA1* [28 (70.0%) of 40] and *RARB* [16 (40.0%) of 40]. In invasive bladder tumors, the most frequent promoter methylation was detected for *THBS1* and *WT1* [both showed in 5 (55.6%) of 9], followed by *BRCA1*, *MSH6*, and *VHL* [all showed in 4 (44.4%) of 9]. In addition, *BRCA1* was the most frequently methylated in both tumor histosubtypes, being methylated in 65.3% of all of the patients analyzed. Methylation of *WT1*, *THBS1*, *MSH6*, *PAX5A*, *VHL*, *BRCA2*, and *CDH13* was higher in muscle-invasive bladder tumors, whereas methylation of *BRCA1*, *RARB*, and *MGMT* was more common in non–muscle-invasive bladder neoplasms (Table 6 and Figure 1B). However, none of the genes analyzed was significantly associated with tumor stage in the validation set. Regarding tumor grade, the more frequent methylated genes in G1 tumors were *BRCA1* and *PAX5A*; in G2 and G3 tumors, they were *BRCA1*, *THBS1*, *RARB*, and *MSH6*; finally, in G3 tumors, *WT1* was an addition to the most commonly methylated genes. Although they were not among the most frequently methylated genes, a significant association was found between tumor grade and the urinary methylation rates of *VHL* (P = 0.003; 99% CI, 0.002 to 0.005), *BRCA2* (P = 0.028; 99% CI, 0.024 to 0.032), and *PTEN* (P = 0.037; 99% CI, 0.032 to 0.042). The receiver operating characteristic curve analyses supported the highest diagnostic accuracies for *WT1* (P = 0.02; 99% CI, 0.0005 to 0.002), *RARB* (P = 0.027; 99% CI, 0.023 to 0.031), *PYCARD* (P = 0.038; 99% CI, 0.033 to 0.043), *GATA5* (P = 0.033; 99% CI, 0.029 to 0.039), *BRCA1* (P = 0.037; 99% CI, 0.032 to 0.041), *TP73* (P =

Table 5. Summary of the AUCs in the Training and Validation Sets of Urinary Specimens

	Training set		Validation set	
Gene name	AUC (95% confidence interval)*	*P* value	AUC (95% confidence interval)*	*P* value
TP73	0.584 (0.451–0.717)	NS	0.651 (0.525–0.776)	0.031
MSH6	0.546 (0.413–0.680)	NS	0.515 (0.383–0.646)	NS
VHL	0.433 (0.299–0.567)	NS	0.614 (0.484–0.745)	NS
RARB	0.686 (0.561–0.807)	0.007	0.650 (0.553–0.774)	0.027
ESR1	0.613 (0.484–0.742)	NS	0.518 (0.382–0.654)	NS
CDKN2A	0.514 (0.378–0.649)	NS	0.606 (0.477–0.735)	NS
PAX5A	0.650 (0.522–0.778)	0.036	0.621 (0.495–0.747)	NS
PTEN	0.622 (0.492–0.752)	NS	0.587 (0.456–0.719)	NS
MGMT	0.561 (0.429–0.694)	NS	0.658 (0.536–0.781)	0.024
MGMT 2	0.533 (0.396–0.670)	NS	0.539 (0.404–0.675)	NS
PAX6	0.617 (0.487–0.747)	NS	0.605 (0.474–0.736)	NS
WT1	0.671 (0.549–0.794)	0.017	0.722 (0.606–0.838)	0.002
CD44	0.589 (0.457–0.721)	NS	0.594 (0.461–0.728)	NS
GSTP1	0.577 (0.446–0.709)	NS	0.614 (0.488–0.740)	NS
ATM	0.614 (0.486–0.743)	NS	0.584 (0.449–0.718)	NS
IGSF4	0.580 (0.449–0.710)	NS	0.639 (0.511–0.767)	NS
CHFR	0.496 (0.361–0.630)	NS	0.600 (0.468–0.733)	NS
BRCA2	0.549 (0.416–0.681)	NS	0.614 (0.486–0.742)	NS
RB1	0.628 (0.499–0.757)	NS	0.610 (0.480–0.740)	NS
RB1 2	0.612 (0.483–0.742)	NS	0.584 (0.450–0.718)	NS
THBS1	0.622 (0.495–0.749)	NS	0.561 (0.429–0.693)	NS
PYCARD	0.565 (0.427–0.702)	NS	0.631 (0.503–0.758)	0.038
CDH13	0.681 (0.558–0.804)	0.011	0.639 (0.513–0.766)	NS
TP53	0.629 (0.493–0.766)	NS	0.591 (0.459–0.724)	NS
BRCA1	0.723 (0.602–0.843)	0.002	0.647 (0.516–0.777)	0.037
STK11	0.463 (0.326–0.599)	NS	0.533 (0.398–0.669)	NS
GATA5	0.606 (0.476–0.735)	NS	0.650 (0.525–0.774)	0.033

AUC, area under the curve; NS, not significant.
*Lower and upper limits are given.

0.031; 99% CI, 0.026 to 0.035), and *MGMT* ($P = 0.024$; 99% CI, 0.022 to 0.030) (Table 5). In summary, the specificities for each gene were similar in both training and validation sets (Table 6). More important, the most frequently methylated genes were confirmed to provide similar diagnostic abilities to identify patients with bladder cancer. The independent test set analysis performed served to confirm the relevance of the hypermethylation of *BRCA1*, *RARB*, and *WT1* in uroepithelial cancer. Overall, these analyses indicated the feasibility of the MS-MLPA assay for bladder cancer diagnostics using noninvasive urinary specimens.

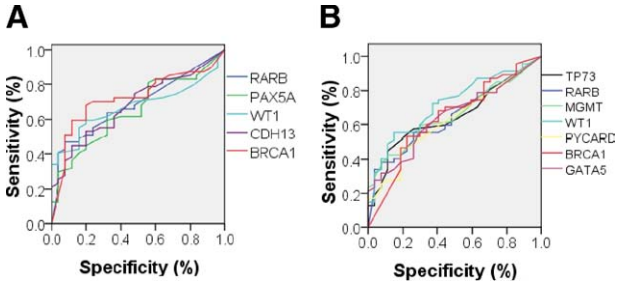

Figure 1. The receiver operating characteristic curves displaying the diagnostic accuracy of the top discriminating genes being analyzed; these genes were differentially methylated between patients with bladder cancer and individuals without the disease, in the training set (**A**) and in the validation set (**B**) of urinary specimens.

Discussion

The novelty of this report deals with the application of a multiplexed methylation technique for a panel of tumor suppressor candidates in bladder cancer. An MS-MLPA was initially tested in cell lines and tissue specimens representing different steps of bladder cancer progression, supporting the panel of the tumor suppressor genes selected to be altered in bladder cancer. It was then optimized for its application in body fluids for noninvasive bladder cancer diagnostics. More important, this study innovates by applying the MS-MLPA technique to urinary specimens. Genes known to be methylated in bladder cancer, such as *BRCA1*, *CDH13*, and *RARB*, were confirmed to be epigenetically modified in the three types of samples analyzed. Moreover, novel candidates, such as *WT1* and *PAX5A*, were revealed as frequently methylated in urinary specimens. Our observations conclude that MS-MLPA is a novel methylation multiplexed tool potentially useful for biological research with *in vitro* samples, histopathological stratification of bladder tumors, and diagnosis in urinary specimens. The latter is clinically relevant because it offers a noninvasive strategy for the clinical management of patients affected with bladder cancer.

Among the high-throughput techniques available for epigenetic alterations assessment, the CpG array represents the major comprehensive platform already applied to identify methylation candidates in bladder cancer.[15] The advantages of the MS-MLPA technique as an alter-

Table 6. Summary of the Sensitivity and Specificity of the Methylation Profiles in the Validation Set of Urinary Specimens

	Controls (n = 25)		Patients (n = 49)		pTa (n = 15)		pT1 (n = 25)		pT2+ (n = 9)		G1 (n = 15)		G2 (n = 16)		G3 (n = 18)	
Gene name	No. of samples*	Specificity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity	No. of samples*	Sensitivity
TP73†	0	100	1	2	0	0	1	4	0	0	0	0	0	0	1	5.5
MSH6†	4	84	16	32.7	6	40	6	24	4	44.4	2	13.3	7	43.7	7	38.9
VHL	1	96	8	16.3	2	13.3	2	8	4	44.4	1	6.7	1	6.2	6	33.3
RARB	2	92	19	38.8	3	20	13	52	3	33.3	5	33.3	7	43.7	7	38.9
ESR1†	0	100	5	10.2	1	6.7	4	16	0	0	0	0	3	18.7	2	11.1
CDKN2A	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAX5A†	1	96	12	24.5	2	13.3	7	28	3	33.3	6	40	1	6.2	5	27.8
PTEN†	1	96	1	2	0	0	0	0	1	11.1	0	0	0	0	1	5.5
MGMT	0	100	7	14.3	3	20	3	12	1	11.1	3	20	2	12.5	2	11.1
MGMT-2	3	88	11	22.5	6	40	5	20	0	0	4	26.7	6	37.5	1	5.5
PAX6	0	100	1	2	0	0	0	0	1	11.1	0	0	0	0	1	5.5
WT1†	1	96	18	36.7	5	33.3	8	32	5	55.5	5	33.3	5	31.2	8	44.4
CD44†	2	92	9	18.4	3	20	4	16	2	22.2	1	6.7	4	25	4	22.2
GSTP1	0	100	1	2	0	0	0	0	1	11.1	0	0	0	0	1	5.5
ATM†	0	100	4	8.2	2	13.3	2	8	0	0	1	6.7	2	12.5	1	5.5
IGSF4†	0	100	6	12.2	4	26.7	2	8	0	0	1	6.7	4	25	1	5.5
CHFR†	0	100	2	4.1	0	0	2	8	0	0	0	0	0	0	2	11.1
BRCA2†	1	96	9	18.4	2	13.3	4	16	3	33.3	2	13.3	1	6.2	6	33.3
RB1	0	100	2	4.1	1	6.7	0	0	1	11.1	0	0	1	6.2	1	5.5
RB1-2	1	96	6	12.2	2	13.3	3	12	1	11.1	1	6.7	1	6.2	4	22.2
THBS1†	3	88	17	34.7	4	26.7	8	32	5	55.5	3	20	7	43.7	7	38.9
PYCARD†	0	100	6	12.2	2	13.3	3	12	1	11.1	1	6.7	2	12.5	3	16.7
CDH13	1	96	11	22.5	2	13.3	6	24	3	33.3	3	20	2	12.5	6	33.3
TP53	1	96	9	18.4	3	20	4	16	2	22.2	1	6.7	5	31.2	3	16.7
BRCA1	9	64	32	65.3	11	73.3	17	68	4	44.4	8	53.3	13	81.2	11	61.1
STK11†	0	100	3	6.1	1	6.7	2	8	0	0	0	0	1	6.2	2	11.1
GATA5†	0	100	10	20.4	2	13.3	7	28	1	11.1	3	20	2	12.5	5	27.8

*Those displaying a methylation ratio higher than 0.3.
†Novel candidates not reported to be methylated in bladder cancer to date.

native for MS-PCR include allowing screening of more than 25 predefined promoter methylation candidates in one single step using a low amount of DNA (100 to 200 ng),[16] which is feasible using DNA extracted from tissue (even in formalin-fixed material)[18]; providing semiquantitative data; and requiring only standard laboratory equipment. Furthermore, the (potentially) troublesome bisulfite conversion of unmethylated cytosines required for MS-PCR[31–39] can be omitted in MS-MLPA using methylation-sensitive digestion. The present study showed that these generally acknowledged advantages can also be translated to investigate methylation in body fluids in a multiplexed manner. Methylation indexes for most probes being studied were consistent and provided interassay reproducibility ranges reliable enough for methylation analysis.

Based on the analysis of matching bladder tumors and urinary specimens of a subset of individuals, it was possible to assess whether the methylation detected in the urinary specimens belonging to patients with bladder cancer could mirror the methylation profiles of bladder tumors. The detection of methylation in matching tumor specimens further validated the relevance of the novel methylated candidates obtained with this gene set. Indeed, many patients with bladder cancer and methylated tumors displayed these epigenetic changes in the paired urine. The use of tissue or urinary specimens precludes some degree of normal tissue mixture and might underscore methylation signatures as positive signals. The high correlation rates among these distinct types of specimens supported the cancer specificity of the methylated candidates. The clinical outcome of the patients whose tumors were analyzed using this technique will reveal if individual tumors behave according to histopathological staging or according to their molecular characterization using this type of multiplexed strategy. The approach thereby offered an opportunity to potentially improve prognostic statements.

A major objective was to interrogate and reliably detect promoter hypermethylation of multiple candidate genes simultaneously using an MS-MLPA panel in urinary specimens with a low amount of starting DNA. Our data revealed that the MS-MLPA assay robustly detected normal and abnormal patterns of methylation in two sets of urinary specimens. The rate of false-positive methylation results was relatively low. Interestingly, three urinary specimens with negative cystoscopy results and altered methylation profiles by MS-MLPA were later diagnosed with initial negative cystoscopy results and altered MS-MLPA results at the same time. The follow-up availability of individuals with negative cystoscopy results and aberrant methylation findings will reveal whether MS-MLPA could be used to predict bladder cancer before the tumor is detectable by the current gold standard diagnostic method. Although the sensitivity found was statistically significant for five of the genes tested, two independent sets of urinary specimens served to validate the clinical relevance of methylation patterns for bladder cancer diagnostics. The extent of methylation in low-grade lesions in our series was higher than the current sensitivity (approximately 30%) of urinary cytology,[29] an observation highlighting the relevance of the profiles as potential noninvasive adjuncts to clinical diagnosis.

The results of the validation set for the top differentiating genes concurred with the main MS-MLPA results in the training set. In addition to the interindividual variation, the differences in sensitivity found between the sets and previous reports describing urinary meth-

ylation for some of the candidates under study (ie, *RARB*,[38] *CDKN2A*,[33,34,36,37,39] *MGMT*,[32,36,39] *GSTP1*,[32,36,39] *CDH13*,[35,38] or *BRCA1*[38]) could be attributed to several issues. Aberrant methylation was defined as needing to meet the cutoff ratio of 30% or greater set by the mathematical algorithm designed to distinguish legitimate methylation peaks. Moreover, the degree of hypermethylation among sets could vary because of the amount of normal cells present in the analyzed sample and the polyclonality of the tumors regarding hypermethylation. Thus, based on the mixed population of cancer and nonneoplastic cells in urine, decreasing the cutoff setting would render improved diagnostic accuracies for each specific gene. Because this is a pilot study to explore the feasibility of MS-MLPA as a high-throughput application to interrogate samples with limited amounts of DNA extracted from body fluids, validation by standard MS-PCR,[38] in which one gene is examined at a time, was restricted because of the latter requiring a high amount of starting DNA material (which is not available for all of the urinary specimens). A discrepancy in the frequency of methylation detected between sets might be attributed, in part, to the type of stages in case groups and controls analyzed, although results and patient distribution between both groups were relatively similar. In addition, differences with previously reported MS-PCR results could be explained by heterogeneity of the promoter methylation for certain genes in bladder cancer carcinomas. This finding could result in absence of complete concordance, explained by the fact that MS-MLPA is only based on a single CpG site compared with an average of four to six CpG sites in MS-PCR assays and heterogeneous methylation patterns that may exist within each individual gene promoter. Because only a small part of the promoter is analyzed by MS-MLPA, it cannot be excluded from additional methylation of nearby CpG islands. The availability of two probes targeting different CpG islands with different methylation ratios for two of the genes analyzed served to highlight the differential methylation and potential consequences of each specific CpG site within a gene. The MS-MLPA ratios may potentially be underestimated because of the presence of normal "contaminating" cells in the tumor and/or urinary sample. However, although the detection of an unmethylated promoter next to methylated sequences is usually disregarded as originating from normal tissue, it could also reflect tumor heterogeneity.

The assay was selected because it contained a set of known or suspected tumor suppressor genes, although they are not bladder cancer specific. Having information of epigenetic alterations reported for several genes included in the MS-MLPA under analyses in bladder tumors, and even in urinary specimens by an independent method,[32–39] served to support our findings in urinary specimens. A correlation of epigenetic aberrations with bladder cancer histopathological variables was reported in numerous studies[8–12,32–39] using techniques other than MS-MLPA. This is explained by an accumulation of genomic damage during tumor progression.[40] Finding more epigenetic aberrations in pT1 tumors compared with pTa lesions represents another level of validation of this concept using our approach.[8–12] Because urinary specimens were prospectively collected, the number of T1 tumors was more frequently represented compared with Ta and T2+ tumors in our series, per the incidence of each stage of the disease.[1] Detecting known aberrations and more methylation alterations in high-grade urinary specimens supports the validity of the approach taken. More important, based on the tumor stages in which methylation was identified in the urinary specimens and in bladder tumors, a sequential line of epigenetic events can be proposed. Methylations of *MSH6*, *RARβ*, and *BRCA1* promoter were identified as early events associated with non–muscle-invasive tumors, even in pTaG1 lesions. The most frequent methylated genes previously reported in urinary specimens of patients with bladder cancer were *BRCA1*,[38] *RARB*,[39] and *CDH13*[38]; these genes also occurred in bladder cancer cell lines and tumors. The detection of distinct methylation profiles between noninvasive and invasive bladder tumors is in concordance with genes previously reported as methylated in bladder cancer by standard MS-PCR,[32–39] despite the fact that different CpG dinucleotides could be targeted by the MS-MLPA and MS-PCR methods. The MS-MLPA method allowed identification of several new and possibly interesting epigenetic alterations (eg, *MSH6*, *WT1*, and *THBS1* genes), serving to gain more insight into the development of bladder carcinomas. To what extent these genes contribute or are functionally involved in the different steps during tumorigenesis and cancer progression remains to be determined. This information will greatly contribute to the design of a clinically optimal biomarker panel in terms of both sensitivity and specificity. These genes represent attractive targets for cancer therapy, given the reversible nature of epigenetic gene silencing. Further studies are warranted to address these critical issues.

This report highlights that the identification of aberrant methylation in promoter regions of cancer genes yields important tumor biomarkers, underscoring a role for epigenetics in tumor pathogenesis. The innovative applicability of MS-MLPA in the three types of samples analyzed contributed to the further understanding of bladder cancer biological features. Moreover, the clinical translational applications of the MS-MLPA platform relate for tumor stratification purposes using tissue material and for noninvasive bladder cancer diagnostics using urinary samples. The utility of MS-MLPA for reliable detection of epigenetic tumor suppressor genes in urinary specimens was a potential diagnostic adjunct per the cross comparison between the independent training and validation sets. The general character of the assay used (with predefined tumor suppressor genes not necessarily specific to any tumor type) suggested the need to investigate regions that would be more relevant for bladder cancer and to develop tumor-specific customized MS-MLPAs for uroepithelial neoplasias. Further research is warranted to establish a more targeted entity-specific combination of genes that may improve the diagnostic performance of the assay and achieve translation of this approach into clinical routine practice. In the near future, the semiquantitative aspect of MS-MLPA may prove to play a role for

early detection and follow-up of patients with bladder cancer and may provide prognostic and/or predictive value for clinical outcome and therapeutic response.

Acknowledgments

We thank all members from the Tumor Markers Group for their technical support and constructive suggestions in the preparation of the manuscript; and all of the members of our clinical collaborators at the different institutions involved in this study for their support in facilitating the urinary and tumor specimens and the clinical information of the bladder cancer cases analyzed in this study.

References

1. Cordon-Cardo C, Cote RJ, Sauter G: Genetic and molecular markers of urothelial premalignancy and malignancy. Scand J Urol Nephrol Suppl 2000, 205:82–93
2. Wolff EM, Liang G, Jones PA: Mechanisms of disease: genetic and epigenetic alterations that drive bladder cancer. Nat Clin Pract Urol 2005, 2:502–510
3. Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002, 3:415–428
4. Feinberg AP, Ohlsson R, Henikoff S: The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006, 7:21–33
5. Costello JF, Fruhwald MC, Smiraglia DJ: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000, 24:132–138
6. Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene 2002, 21: 5427–5440
7. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003, 349:2042–2054
8. Hu YC, Sidransky D, Ahrendt SA: Molecular detection approaches for smoking associated tumors. Oncogene 2002, 21:7289–7297
9. Kim WJ, Quan C: Genetic and epigenetic aspects of bladder cancer. J Cell Biochem 2005, 95:24–33
10. Zhang Z, Karam J, Frenkel E, Sagalowsky A, Hsieh JT: The application of epigenetic modifiers on the treatment of prostate and bladder cancer. Urol Oncol 2006, 24:152–160
11. Baffa R, Letko J, McClung C, LeNoir J, Vecchione A, Gomella LG: Molecular genetics of bladder cancer: targets for diagnosis and therapy. J Exp Clin Cancer Res 2006, 25:145–160
12. Gonzalgo ML, Datar RH, Schoenberg MP, Cote RJ: The role of deoxyribonucleic acid methylation in development, diagnosis, and prognosis of bladder cancer. Urol Oncol 2007, 25:228–235
13. Yan PS, Perry MR, Laux DE, Asare AL, Caldwell CW, Huang TH: CpG island arrays: an application toward deciphering epigenetic signatures of breast cancer. Clin Cancer Res 2000, 6:1432–1438
14. Ching TT, Maunakea AK, Jun P, Hong C, Zardo G, Pinkel D, Albertson DG, Fridlyand J, Mao JH, Shchors K, Weiss WA, Costello JF: Epigenome analyses using BAC microarrays identify evolutionary conservation of tissue-specific methylation of SHANK3. Nat Genet 2005, 37:645–651
15. Aleman A, Adrien L, Lopez-Serra L, Cordon-Cardo C, Esteller M, Belbin TJ, Sanchez-Carbayo M: Identification of DNA hypermethylation of SOX9 in association with bladder cancer progression using CpG microarrays. Br J Cancer 2008, 98:466–473
16. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G: Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002, 30:e57
17. Nygren AO, Ameziane N, Duarte HM, Vijzelaar RN, Waisfisz Q, Hess CJ, Schouten JP, Errami A: Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. Nucleic Acids Res 2005, 33:e128
18. Worsham MJ, Chen KM, Meduri V, Nygren AO, Errami A, Schouten JP, Benninger MS: Epigenetic events of disease progression in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2006, 132:668–677
19. Jeuken JW, Cornelissen SJ, Vriezen M, Dekkers MM, Errami A, Sijben A, Boots-Sprenger SH, Wesseling P: MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas. Lab Invest 2007, 87:1055–1065
20. Berkhout M, Nagtegaal ID, Cornelissen SJ, Dekkers MM, van de Molengraft FJ, Peters WH, Nagengast FM, van Krieken JH, Jeuken JW: Chromosomal and methylation alterations in sporadic and familial adenomatous polyposis-related duodenal carcinomas. Mod Pathol 2007, 20:1253–1262
21. Scott RH, Douglas J, Baskcomb L, Nygren AO, Birch JM, Cole TR, Cormier-Daire V, Eastwood DM, Garcia-Minaur S, Lapunzina P, Tatton-Brown K, Bliek J, Maher ER, Rahman N. Methylation-specific MLPA (MS-MLPA) robustly detects and distinguishes 11p15 abnormalities associated with overgrowth and growth retardation. J Med Genet 2008, 45:106–113
22. Henken FE, Wilting SM, Overmeer RM, van Rietschoten JG, Nygren AO, Errami A, Schouten JP, Meijer CJ, Snijders PJ, Steenbergen RD: Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. Br J Cancer 2007, 97:1457–1464
23. Chen K, Sawhney R, Khan M, Benninger MS, Hou Z, Sethi S, Stephen JK, Worsham MJ: Methylation of multiple genes as diagnostic and therapeutic markers in primary head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2007, 133:1131–1138
24. Hess CJ, Errami A, Berkhof J, Denkers F, Ossenkoppele GJ, Nygren AO, Schuurhuis GJ, Waisfisz Q: Concurrent methylation of promoters from tumor associated genes predicts outcome in acute myeloid leukemia. Leuk Lymphoma 2008, 49:1132–1141
25. Ewald C, Hofmann T, Kuhn SA, Deufel T, Beetz C, Kalff R: Methylation-specific multiplex ligation-dependent probe amplification in meningiomas. J Neurooncol 2008, 90:267–273
26. Gylling A, Ridanpää M, Vierimaa O, Aittomäki K, Avela K, Kääriäinen H, Laivuori H, Pöyhönen M, Sallinen SL, Wallgren-Pettersson C, Järvinen HJ, Mecklin JP, Peltomäki P: Large genomic rearrangements and germline epimutations in Lynch syndrome. Int J Cancer 2009, 124: 2333–2340
27. Buyru N, Altinisik J, Ozdemir F, Demokan S, Dalay N: Methylation profiles in breast cancer. Cancer Invest 2009, 27:307–312
28. Sanchez-Carbayo M, Socci ND, Charytonowicz E, Lu M, Prystowsky M, Childs G, Cordon-Cardo C: Molecular profiling of bladder cancer using cDNA microarrays: defining histogenesis and biological phenotypes. Cancer Res 2002, 62:6973–6980
29. Kirkali Z, Chan T, Manoharan M, Algaba F, Busch C, Cheng L, Kiemeney L, Kriegmair M, Montironi R, Murphy WM, Sesterhenn IA, Tachibana M, Weider J: Bladder cancer: epidemiology, staging and grading, and diagnosis. Urology 2005, 66:4–34
30. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C: Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J Clin Oncol 2006, 24:778–789
31. Huster WJ, Louv WC: Demonstration of the reproducibility of treatment efficacy from a single multicenter trial. J Biopharm Stat 1992, 2:219–238
32. Chan MW, Chan LW, Tang NL, Tong JH, Lo KW, Lee TL, Cheung HY, Wong WS, Chan PS, Lai FM, To KF: Hypermethylation of multiple genes in tumor tissues and voided urine in urinary bladder cancer patients. Clin Cancer Res 2002, 8:464–470
33. Friedrich MG, Weisenberger DJ, Cheng JC, Chandrasoma S, Siegmund KD, Gonzalgo ML, Toma MI, Huland H, Yoo C, Tsai YC, Nichols PW, Bochner BH, Jones PA, Liang G: Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients. Clin Cancer Res 2004, 10:7457–7465
34. Dulaimi E, Uzzo RG, Greenberg RE, Al-Saleem T, Cairns P: Detection of bladder cancer in urine by a tumor suppressor gene hypermethylation panel. Clin Cancer Res 2004, 10:1887–1893
35. Yates DR, Rehman I, Meuth M, Cross SS, Hamdy FC, Catto JW: Methylational urinalysis: a prospective study of bladder cancer patients and age stratified benign controls. Oncogene 2006, 25:1984–1988
36. Hoque MO, Begum S, Topaloglu O, Chatterjee A, Rosenbaum E, Van Criekinge W, Westra WH, Schoenberg M, Zahurak M, Goodman SN,

Sidransky D: Quantitation of promoter methylation of multiple genes in urine DNA and bladder cancer detection. J Natl Cancer Inst 2006, 98:996–1004
37. Pu RT, Laitala LE, Clark DP: Methylation profiling of urothelial carcinoma in bladder biopsy and urine. Acta Cytol 2006, 50:499–506
38. Yu J, Zhu T, Wang Z, Zhang H, Qian Z, Xu H, Gao B, Wang W, Gu L, Meng J, Wang J, Feng X, Li Y, Yao X, Zhu J: A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer. Clin Cancer Res 2007, 13:7296–7304
39. Phé V, Cussenot O, Rouprêt M: Interest of methylated genes as biomarkers in urothelial cell carcinomas of the urinary tract. BJU Int 2009, 104:896–901
40. Esteller M: Relevance of DNA methylation in the management of cancer. Lancet Oncol 2003, 4:351–358